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IDENTIFICATION OF FUNGI AND THEIR SECONDARY METABOLITES FROM WIND CAVE

A Thesis Submitted

in Partial Fulfillment

of the Requirements for the Designation

University Honors

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University of Northern Iowa

December 2021

This Study by: Kyle Biscoglia

Entitled: Identification of Fungi and Their Secondary Metabolites

has been approved as meeting the thesis or project requirement for the Designation University Honors

12/13/2021 _Dr. Kirk Manfredi_____

Date Dr. Kirk Manfredi, Honors Thesis Advisor, Department of Chemistry

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Date Dr. Jessica Moon, Director, University Honors Program

Abstract

Eighteen fungal samples were isolated from samples of water collected in Wind Cave, South Dakota. Ten of the samples underwent chemical extraction, a growth inhibition test, and were analyzed using proton nuclear magnetic resonance spectroscopy and liquid chromatography-mass spectroscopy. Structural analysis and identification of one secondary metabolite that demonstrated antimicrobial growth inhibition was performed. All eighteen of the fungal samples were able to be identified to the genus level using the ITS region of their DNA, however confident identification of the species level was more difficult to attain just looking at one portion of the sequence.

Introduction

As new fungal species and their secondary metabolites are discovered and isolated, it is increasingly important that fungi are identified and cataloged correctly. The identification of new fungi allows the scientific community to grow. The identification and isolation of new secondary metabolites produced by fungi can be useful for a variety of reasons. One such reason that is pertinent to this project is the fact that these natural products can be used to develop antibiotic drugs. This idea guides on aspect of this research project, which is to purify and identify secondary metabolites that may have a therapeutic use.

The problem this research addresses stems from the fact that due to the constant use of antibiotics antimicrobial drug resistance has become more and more common because of reasons such as the misuse of antibiotics. Examples of misuse include taking antibiotics for viral infections, and not completing the full dose of prescribed antibiotics[1]. It is important to completely follow provider's instructions regarding antibiotic treatments. As the number of antibiotic resistant bacteria strains increases, the effects and efficiency of many common antibiotics decreases. Research into the secondary metabolites produced by various organisms, including fungi, provides a chance to develop new therapeutic drugs that pathogens are not resistant to.

Secondary metabolites are natural products produced by organisms that are not linked to their growth, reproduction, and reproduction. Instead, these natural compounds assist an organism by helping it maintain homeostasis, or steady conditions, by serving as competitive weapons, metal transporting agents, agents of symbiosis between microbes and higher animals, sexual hormones, and as differentiation effecters[2]. Oftentimes, these secondary metabolites have a different purpose in the organism that produces them than its use by humans.

One common example of a secondary metabolite is caffeine. Caffeine is produced by plants in the *Coffea* genus. Plants in this family use caffeine to ward off insects and other pests that would eat its leaves and beans. However, humans use caffeine as a psychoactive drug to prevent the onset of drowsiness. Similarly, the function of secondary metabolites isolated from the fungal samples may serve a different purpose in that organism than what they are used for by humans.

To expedite the screening process of the fungal samples, a two-pronged approach can be utilized. This approach involves identifying the fungal sample through DNA sequencing. Once the fungal sample has been identified research on the sample can be done to see if the fungi has been well studied and research

[1] Bush, K., Courvalin, P., Dantas, G. et al. Tackling antibiotic resistance. Nat Rev Microbiol **9,** 894–896 (2011). https://doi.org/10.1038/nrmicro2693

[2] Demain, Arnold L., and Aiqi Fang. "The natural functions of secondary metabolites." *History of modern biotechnology I* (2000): 1-39.

has been done on its natural products, or if the sample has not had much research done. In this case, more attention can be devoted to the samples that have not been researched as much and are therefore better candidates for producing a new, antimicrobial secondary metabolite.

Working in tandem with the secondary metabolite portion of the project, another main objective of this project was focused on correctly identifying, and discovering, fungal species using the Internal Transcribed Spacer (ITS) region of their DNA[3]. This portion of the DNA acts as a barcode in unique fungal species and allows them to be identified with ease. The ITS region is shown below in Figure 1, along with the location of the primers used to create the sequences.

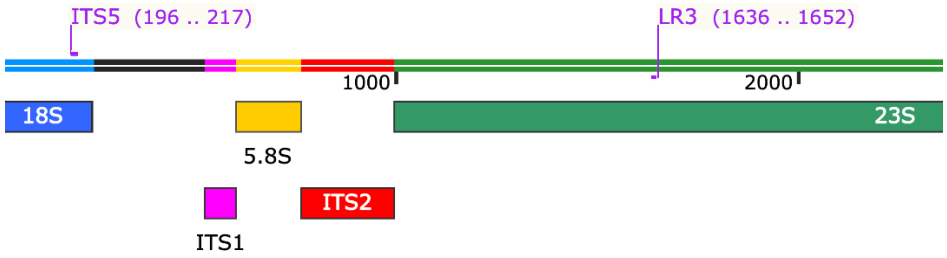

Figure 1. The ITS region sequenced for fungal sample identification[4].

To sequence the ITS region of the fungal DNA a forward and reverse primer were needed. For each sample, the ITS5 and LR3 primers were used to generate DNA sequences that were about 1,500 base pairs in length. The sequences could then be run through a variety of fungal DNA databases to generate matches for the genus and species in question. The databases used to identify the fungal samples are seen below in Figure 2.

Database Name	URL
RefSeq Target Loci	http://www.ncbi.nlm.nih.gov/refseq/targetedloci/
Barcode of Life	http://www.boldsystems.org
UNITE, User-friendly Nordic ITS Ectomycorrhiza database	https://unite.ut.ee/

Figure 2. Databases used for fungal identification.

[3]Schoch, Conrad L., et al. "Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi." *Proceedings of the National Academy of Sciences* 109.16 (2012): 6241-6246.

[4]Raja, Huzefa A., et al. "Fungal identification using molecular tools: a primer for the natural products research community." *Journal of natural products* 80.3 (2017): 756-770.

This project focuses on fungal samples collected from Wind Cave, South Dakota. Wind Cave was chosen as the location for this project because of it is the location of an ongoing project funded by a grant through NASA*. Additionally, Wind cave shows relative isolation from other areas. This increases the probability that a fungal species exist that is undiscovered by the scientific community. Another reason Wind Cave, and specifically Calcite Lake, was chosen as the location for this project was because it is relatively uncontaminated by humans compared to other caves in the Midwest Wind Cave is thought to have anywhere from 400 km – 1800 km of surveyable passages, with a maximum of 40% of the passages currently defined[5]. This allows the unique opportunity to identify fungal samples that have been living in the cave undisturbed for many years. This again increases the probability of finding a new fungal species as well as providing the unique opportunity to see what the unaltered fungal makeup of the cave is.

*This project/material is based upon work supported by the Iowa Space Grant Consortium under NASA Award No. 80NSSC20M0107.

Experimental

To begin this project, water samples were collected from Calcite Lake in Wind Cave South Dakota. The water samples collected from the cave were brought back to the lab in Cedar Falls, Iowa and then plated on two different mediums for growth. The two different mediums that were used were potato dextrose agar (PDA) and yeast extract peptone dextrose (YPD). These two media were used to ensure that any fungal samples present in the water samples would be able to grow. In addition to using two media, samples were placed in light and dark conditions for each medium.

After growth had occurred on the plates, taking anywhere between 1-2 weeks, the different types of fungi were isolated onto their own plates so that they could be studied further individually. At this point, fungi were separated primarily via visual methods such as color and texture. This process involved using a sterile inoculating loop and transferring a portion of a fungal sample to a fresh plate with the same medium and growing it in the same conditions. If a replated sample showed contamination after growth, then the process was repeated until plates were made that had no other bacterial or fungal contamination.

After isolating fungal samples onto their own unique plates, they could be grown in a rice culture to extract their secondary metabolites. To do this, brown rice was washed and autoclaved with water to ensure there was no contamination. After the rice cooled, a sterile inoculating loop was used to transfer a small amount of the fungi from a plate containing a purified sample to the flask containing rice. The flask was sealed with wool and a foil lid and placed in the dark to grow (at least 3 weeks).

As the rice cultures were growing, DNA extraction was performed on the purified plates. To do this a DNA kit was used called the E.Z.N.A. Fungal DNA Mini Kit produced by Omega Norcross. This kit came with a variety of protocols to use for different scenarios. The method used for all the DNA extractions followed the “fresh or frozen” method created for extracting DNA from either fresh or frozen fungal samples. For each unique fungi, two DNA samples were extracted. After the DNA extraction, the DNA concentration was measured using a NanoDrop. This instrument also helped to measure the purity of the DNA samples. The next step for DNA analysis was polymerase chain reaction (PCR) to amplify a target portion of the DNA. The DNA portion amplified by this process was the ITS region of the fungal DNA. The ITS5 and LR3 res used ensured that this was the specific portion of DNA that would be amplified. The mixture created for the PCR combined 1 µL of DNA, 1 µL of each primer, 5 µL of a 10x Taq buffer, 5 µL of a 10x BSA solution, 1 µL of a 50 mM $MgCl_2$ mix, 1 µL of a 10 mM dNTP mix, 1 µL of Taq DNA polymerase, and 31 µL of water. The Initial denaturation step occurred at 94° for 2 minutes.

[5]Horrocks, Rodney D., and Bernard W. Szukalski. "Using geographic information systems to develop a cave potential map for wind cave, South Dakota." *Journal of Cave and Karst Studies* 64.1 (2002): 63-70.

This was followed by 35 cycles at 50° and then a final hold at 72°. After PCR was completed, gel electrophoresis was performed using a 2% agarose gel to ensure that the DNA had been successfully amplified. The samples for gel electrophoresis were created by combining 1 µL of loading dye with 5 µL the amplified sample. A successful amplification would produce bands on the agarose gel that were about 1,500 base pairs in length.

The next step in the DNA identification process was purifying the DNA samples to get rid of contaminants and extra reagents from the PCR reaction. This was done using the GeneJET PCR Purification Kit produced by Thermo Fisher Waltham. After purification, according to the kit protocol the NanoDrop was again used to measure the final Nucleic Acid concentration. This concentration was recorded for each DNA sample, and the samples, along with the primers, were sent to the Iowa State University DNA Biotech Facility in Ames, Iowa for sequencing. The sequences were then returned for both the forward and reverse primers. The largest matches for these sequences were generated by looking at the results and comparing the chromatograms received with the data. These longest hits were put into DNA databases to create matches. The best matches were recorded along with the percent match that they were. The genera of the samples were used to create a phylogenetic tree highlighting the evolutionary relationships of the samples.

After the identification step was completed, research could be done on the matching hits from the DNA databases to see if the fungi matched produced any notable compounds. This was used to guide the extraction process. At this point in the research, the rice cultures started earlier in the project were ready for extraction, although this process was spread out so that the number of extractions each week was manageable. Before each extraction, the fungi growing in the rice culture was transferred onto a plate so that it could be kept and regrown if necessary.

The extraction process followed an altered version of the Kupchan scheme[6] described below. To start the extraction process 50 mL of chloroform and 50 mL of methanol were added to the culture and stirred up to mix the secondary metabolites. This mixture was placed on a shaker overnight. The next morning 75 mL of water and 45 mL of chloroform was added, and the mixture was filtered to separate the liquids from the solids using vacuum filtration. The filtrate was transferred into a separatory funnel and the bottom layer, the chloroform, was collected. Another fraction was collected using 45 mL of chloroform. This fraction was labeled the crude organic. The remaining water was called the crude aqueous.

[6] Kupchan, S. Morris. "Recent advances in the chemistry of terpenoid tumor inhibitors." *Pure and Applied Chemistry* 21.2 (1970): 227-246.

The crude organic fraction was put on the rotary evaporator, to remove most of the solvent. The fraction was then placed on the vacuum pump to remove the last traces of the solvent. The mass of the dried product was recorded, and a small sample was saved for biological testing. The remaining organic product was put back into solution with 54 mL of methanol and 6 mL of water. Three sets of extractions were performed using 25 mL of hexane. The hexane fraction was put on the rotary evaporator, put on the vacuum pump to remove any traces of the solvent, and weighed. approximately 2 mg was saved for biological testing and the rest was stored in a labeled vial for further analysis. The fraction remaining after the hexane extraction was extracted again after adding 17 mL of water. This time 20 mL of chloroform was used three times. The chloroform fraction was then placed on the rotary evaporator as before and then the vacuum pump to remove the solvent. approximately 2 mg was saved for biological testing and the rest was stored in a labeled vial for further analysis. The fraction remaining after the chloroform extraction was placed on the rotary evaporator to remove the methanol. After this only water remained. A final extraction of what was the crude organic fraction was performed twice using 25 mL of ethyl acetate each time. The ethyl acetate was then placed on the rotary evaporator as before and then the vacuum pump to remove the solvent. Approximately 2 mg was saved for biological testing and the rest was stored in a labeled vial for further analysis. The remaining extract was discarded.

The crude aqueous fraction was put on the rotary evaporator until all the methanol had evaporated and only water was left. An extraction was then performed twice using 50 mL of ethyl acetate each time. This ethyl acetate fraction was then placed on the rotary evaporator as before and then the vacuum pump to remove the solvent. approximately 2 mg was saved for biological testing and the rest was stored in a labeled vial for further analysis. The leftover water was freeze-dried and then approximately 2 mg was saved for biological testing.

Immediately following the extraction process High-Pressure Liquid Chromatography (HP-LC) was performed on the ethyl acetate from the aqueous and the chloroform samples. The chromatogram produced was used to create a rough idea of the different compounds present in each sample.
To perform HP-LC methanol was added to the samples. The solution was then transferred into an HP-LC vial so that no more than 300 µL was in the new vial and it contained around 2 mg of sample. The samples were run under a method that gradually changed the solvent from 80% methanol and 20% water to 100% methanol over 14 minutes.

As a result of the extraction process, each rice culture produced 6 samples to be for biological testing: the crude organic, the hexane, chloroform, the ethyl acetate from the organic, the ethyl acetate from the aqueous, and the dried water.

Before biological testing, plates containing bacteria had to be made. To do this, the day before a test 20 mL of soy broth was put into a conical tube along with bacteria. Four types of bacteria were used for the biological tests: Candida albicans, Escherichia coli, Bacillus subtilis, and Staphylococcus aureus. These conical tubes were placed in an incubated shaker overnight. The next day, in smaller conical tubes, 9 mL of fresh soy broth was mixed with 1 mL of the bacteria-rich broth 4-5 hours before the biological test was to be started. These tubes were placed in an incubated shaker. This was done to ensure the bacteria were in their log phase for the next step, which was plating. To do this, the broth from the small tubes was poured over soy plates in a sterile environment. The excess liquid was poured into a bleach solution to kill the bacteria and disposed of appropriately. The plates were labeled with the bacteria they had on them and then ready for testing.

To perform the biological test, dimethyl sulfoxide (DMSO) was added to the samples until it reached a concentration of 2 mg/ µL. At least 16 µL of the solution was required. Next, 4 µL of this solution was then pipetted onto four separate filter paper discs cut out with a paper hole punch and placed on separate plates containing bacteria. This process was repeated for all the samples so that each plate of bacteria had filter paper discs on it spaced out every 2 cm. To ensure the accuracy of the results a positive and negative control were used. The negative control was a filter paper disc with 4 µL of DMSO on it. The positive control was half of a tetracycline disc, a known antimicrobial compound that inhibits the growth of all the bacteria used for the test. The plates were then placed in the incubator overnight to grow. The following morning the results of the experiment could be recorded. Any filter paper disc that had a ring around it where no bacteria had grown was noted, as well as what bacteria it was that had been inhibited.

Following the growth inhibition assay, analysis was performed on the samples that showed antimicrobial activity. The next step was to perform proton nuclear magnetic resonance (NMR) on the samples that showed antimicrobial activity or interesting HP-LC chromatograms. This process returns spectra that can be analyzed to help determine the structure of the molecules tested. The purer the sample is that undergoes NMR, the more helpful and clear the spectrum is. To do this, 400 µL of deuterated chloroform was added to the sample, and it was vortexed. If the sample dissolved in solution, then an additional 300 µL of deuterated chloroform was added. If it did not dissolve, then 300 µL of deuterated methanol was

added and the solution was mixed. If there was still undissolved substrate then the solution was put through a filter. The sample was transferred to a clean NMR tube and proton NMR was performed.

In addition to getting a rough idea of the compounds present in each sample, HP-LC was also used to separate compounds of interest from other compounds based off retention time, which is affected by polarity. Separating the samples allowed for more biological testing to be performed to work to isolate the specific compound of interest. Further isolation of these compounds allowed for more accurate structural analysis of the compound as well as more opportunities to observe the growth inhibition effects of each compound.

The final step involved in the structural analysis of the compounds was liquid chromatography-mass spectroscopy (LC-MS). This was performed on any samples of interest. The samples were prepared via the same method as the HP-LC method listed above. The chromatograms produced by the HP-LC and the LC-MS and the spectra produced via proton NMR were all used for structural analysis.

Results

Figure 3 below shows the first seventeen isolated fungal samples from Wind Cave. These fungal samples were able to be isolated from their initial plates and regrown. Each of these plates acted as the starting source for both the DNA isolation and sequencing and the secondary metabolite extraction.

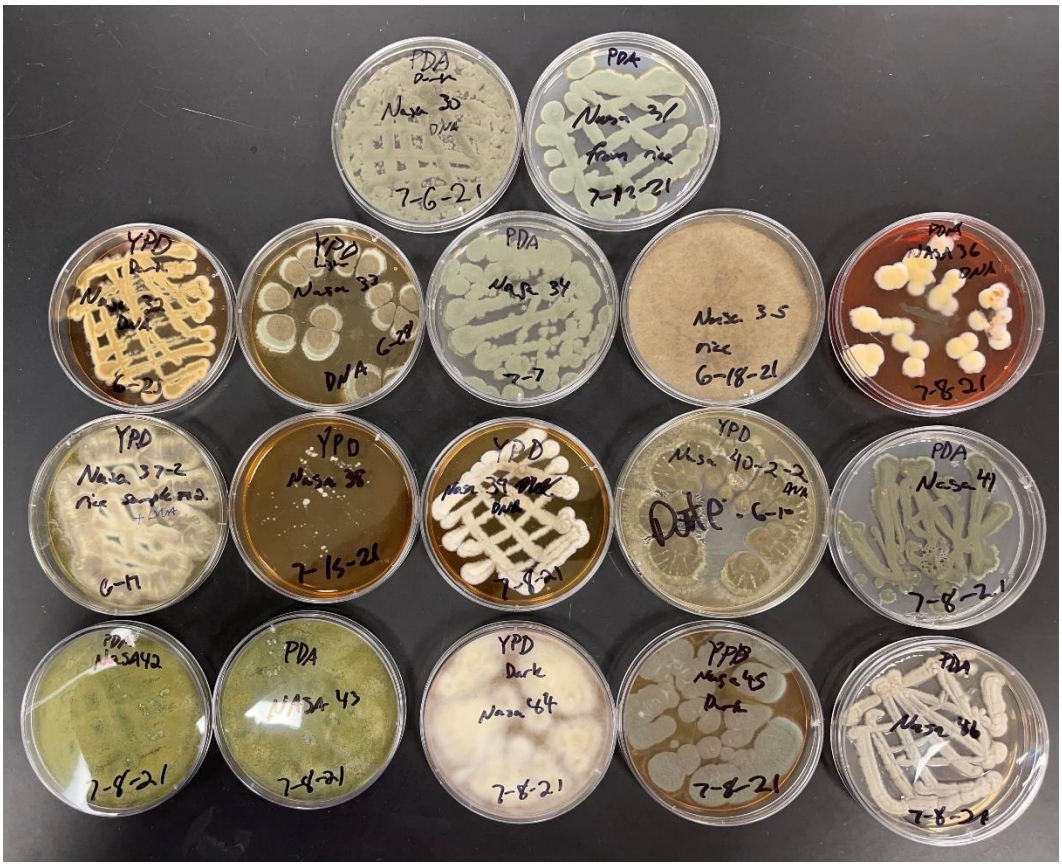

Figure 3. Isolated fungal samples taken from Calcite Lake in Wind Cave, South Dakota. The first row of the plates contains NASA 30 and 31, the second row contains NASA 32-36, the third row contains NASA 37-41, and the fourth row contains NASA 42-46. NASA 47 is not pictured.

Figure 4 below shows a table of all eighteen identified fungal samples as well as what initial Calcite Lake sample they came from. The samples were able to be confidently identified to the genus level for all eighteen of the samples. However, confident identification down to the species level will require sequencing of other regions of the DNA.

Sample Location	Assigned Name	Identified Genus
Calcite Lake 2	NASA 30	*Penicillium*
	NASA 31	*Penicillium*
	NASA 32	*Aspergillus*
	NASA 33	*Cladosporium*
	NASA 34	*Penicillium*
	NASA 35	*Absidia*
	NASA 36	*Aspergillus*
Calcite Lake 3	NASA 37	*Penicillium*
	NASA 38	*Pseudogymnoascus*
Calcite Lake 4	NASA 39	*Penicillium*
	NASA 40	*Penicillium*
	NASA 41	*Talaromyces*
Calcite Lake 6	NASA 47	*Penicillium*
Calcite Lake 7	NASA 42	*Trichoderma*
	NASA 43	*Trichoderma*
Calcite Lake 8	NASA 44	*Chaetomium*
	NASA 45	*Penicillium*
Calcite Lake 9	NASA 46	*Pseudogymnoascus*

Figure 4. Identification of all eighteen fungal isolates.

Figure 5 below shows a phylogenetic tree of all eighteen fungal samples for which DNA was isolated and sequenced. This tree shows the genetic relationship between all the fungi samples. The proximity of the branches represents the similarity in the genetic sequences of the samples; however, the relationship is not proportional. Therefore, it is expected that samples belonging to the same genus would be closer to one another on the tree.

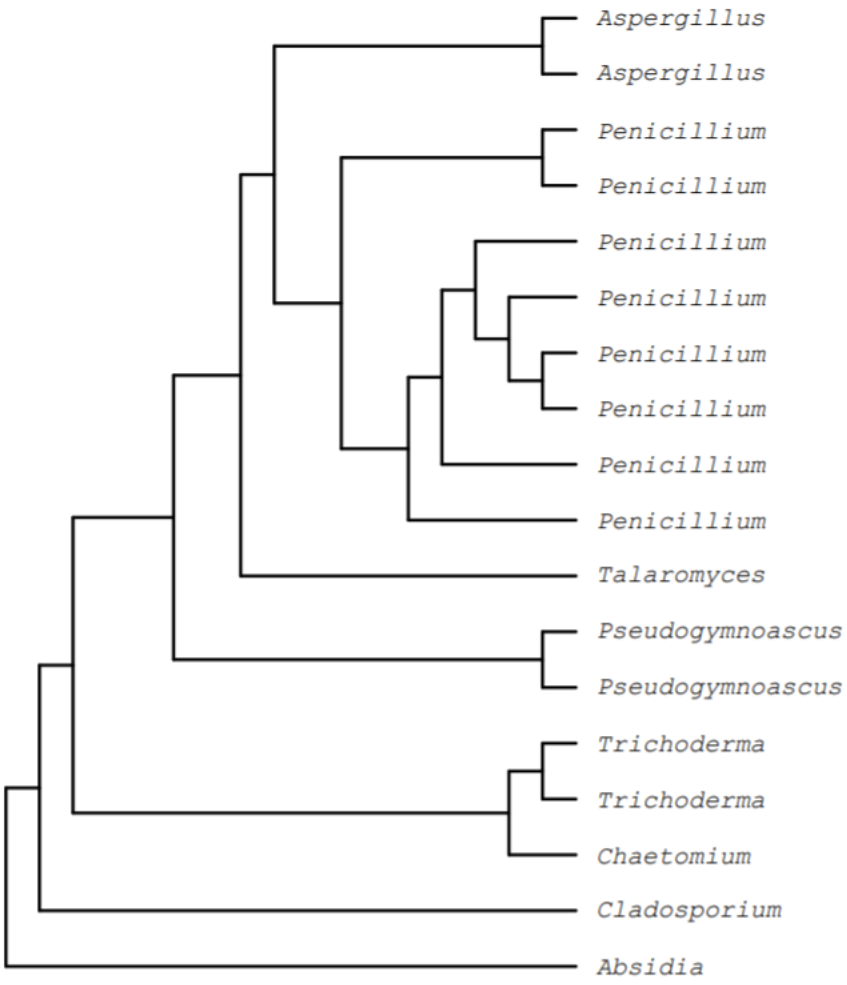

Figure 5. Phylogenetic tree of all eighteen fungal isolates.

Structural identities of purified metabolites

Extraction of multiple samples that demonstrated antimicrobial growth inhibition was successfully performed. However, after extracting the initial sample and trying to isolate the compound that possessed antimicrobial properties, not much of the compound was able to be purified for many of the samples. The lack of purified sample made the structural analysis portion of the project difficult, as not enough of the sample was available for complete analysis through NMR or LC-MS. However, structural analysis was performed on two separate compounds taken from NASA 36 and NASA 29. NASA 29 is believed to be the same sample as NASA 38 and NASA 46.

NASA 36 was identified as an *Aspergillus* and after extraction showed moderate growth inhibition against *Candida albicans*. Preparative HPLC was used to purify the compound that showed this activity. LC-MS was performed, and the compound was determined to have a molecular weight of 371.1147 and a likely chemical formula of $[C_{20}\,H_{19}\,O_7 - H]^-$. Next, structural analysis techniques were used to determine the structure of the antimicrobial compound. Figure 6 shown below is the proton NMR spectrum for the compound isolated from NASA 36. This spectrum was used to determine the location of protons in the compounds structure. Figure 7 shown below is the carbon NMR from the same compound. This spectrum is used to identify unique carbons in the compound. Figure 8 below is a HSQC spectrum which is used to determine proton-carbon bond correlations. Figure 9 below is a HMBC spectrum which shows correlations between protons and carbons separated by two or 3 bonds. Using all of these structural analysis techniques the structure and identity of this compound was able to be confidently determined. This compound, known as averantin, was isolated from NASA 36. Figure 10 shown below shows the structure of averantin.

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[7] Bennett, J. W., et al. "Identification of averantin as an aflatoxin B1 precursor: placement in the biosynthetic pathway." *Applied and environmental microbiology* 39.4 (1980): 835-839.

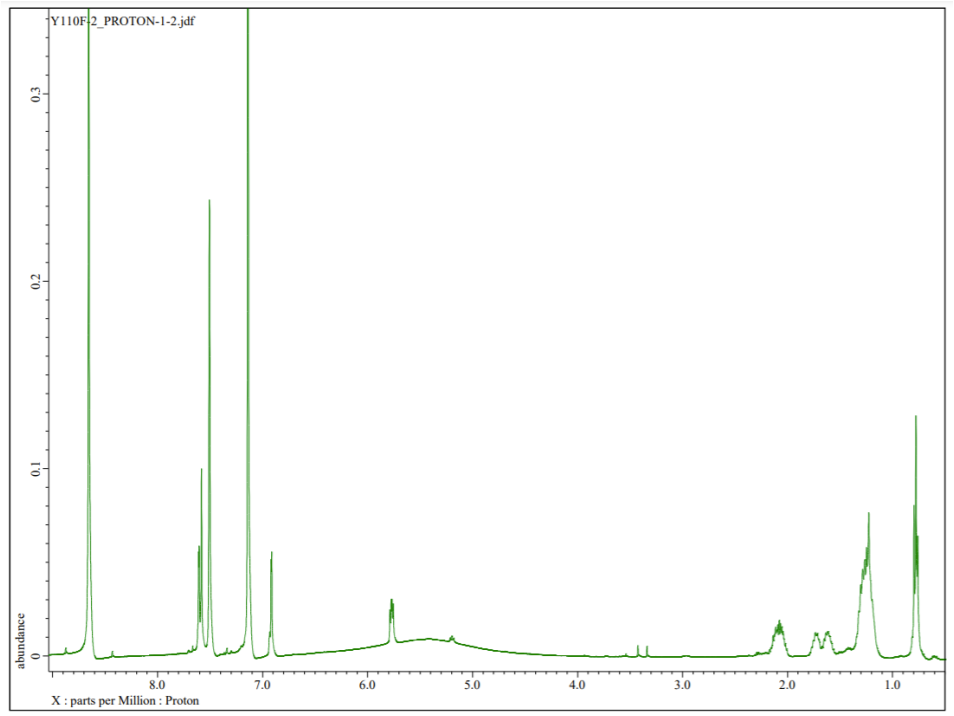

Figure 6. Proton NMR of averantin

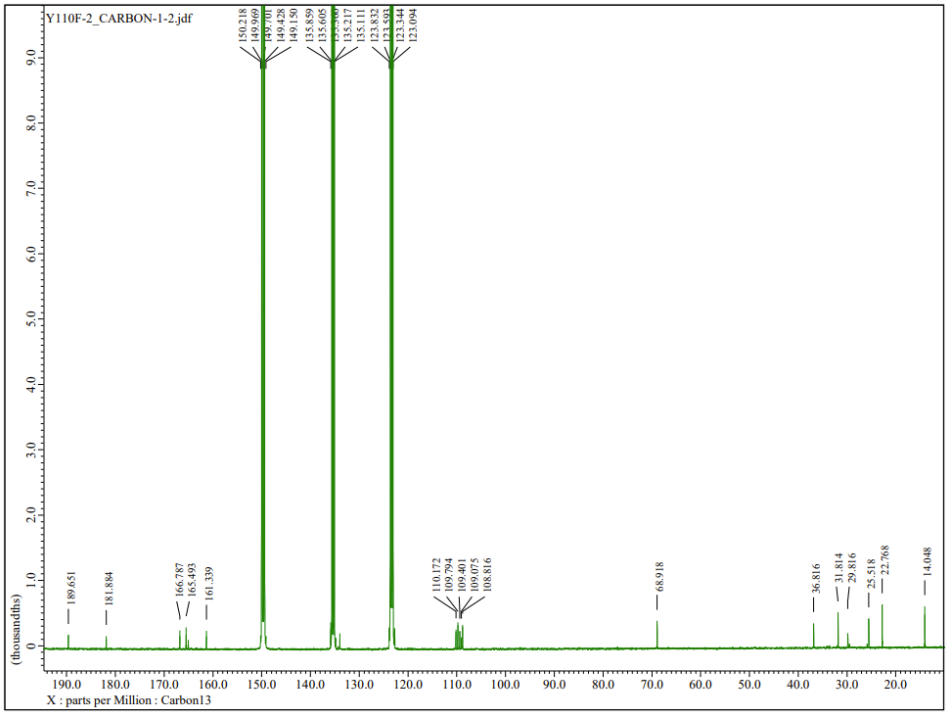

Figure 7. Carbon NMR spectrum of averantin

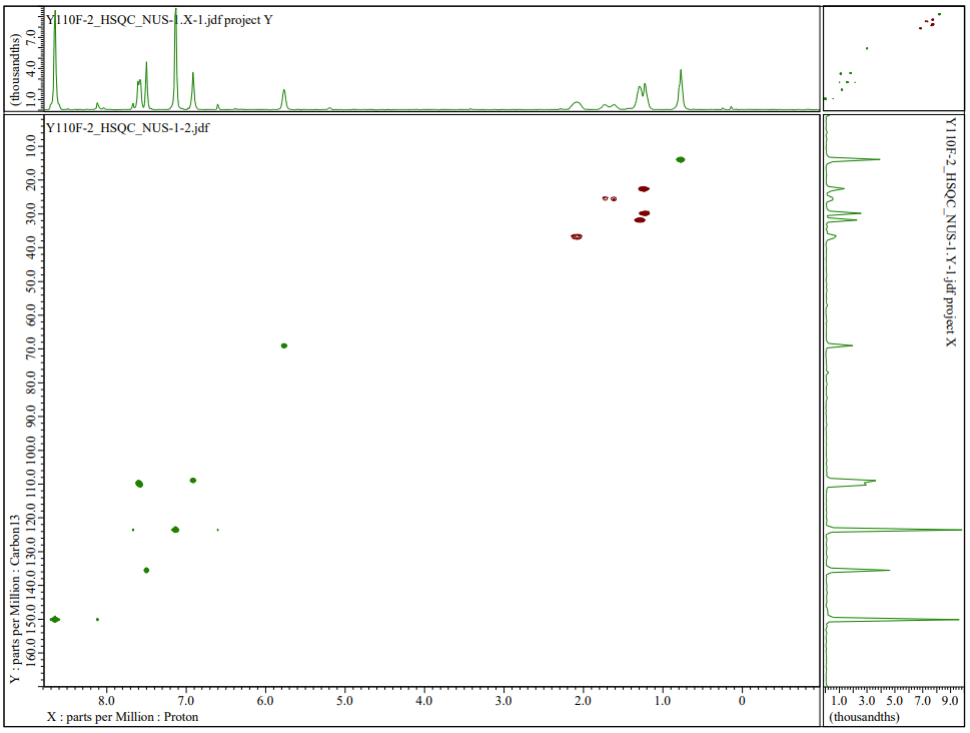

Figure 8. HSQC spectrum of averantin

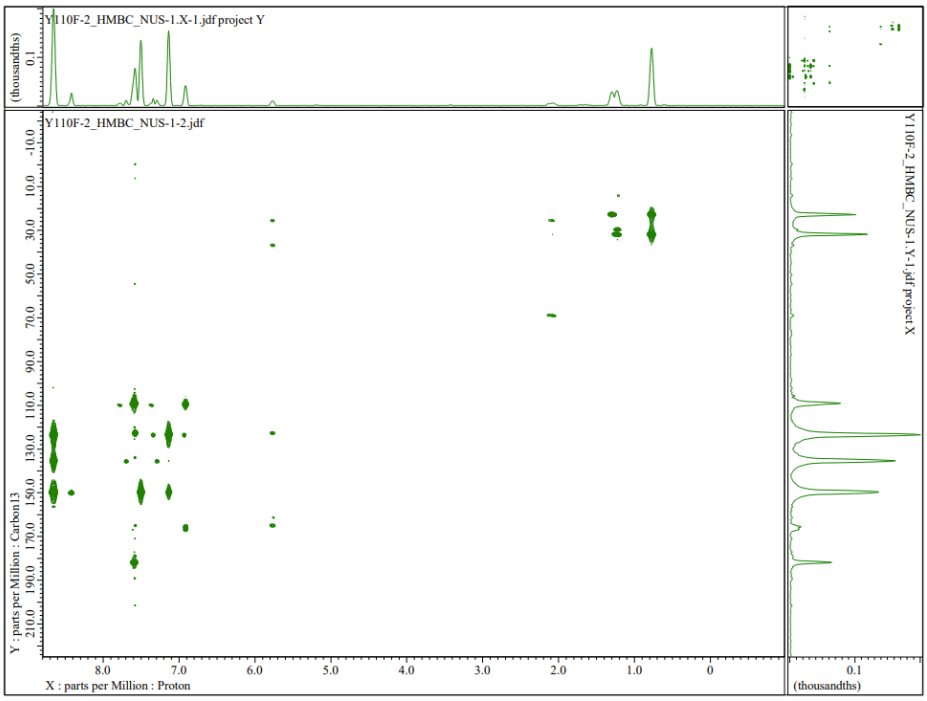

Figure 9. HMBC spectrum of averantin

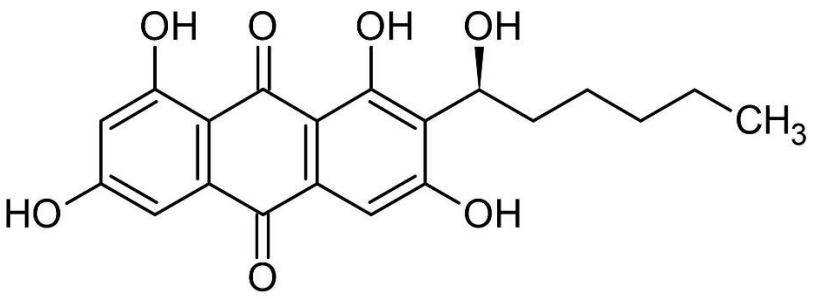

Figure 10. Structure of averantin, a compound isolated from NASA 36 that demonstrated antimicrobial growth inhibition.

Additionally, a compound was isolated from NASA 29, a sample taken from a previous trip to wind cave. The sample belongs to the genus *Pseudogymnoascus*. This sample is believed to be the same as NASA 38 and 46, but further DNA testing is required to conclude this confidently. The *Pseudogymnoascus* genus contains a species, *Pseudogymnoascus destrucans*, that causes white nose syndrome in bats[8]. This caused an initially interest in the sample and analysis was performed on the sample. LC-MS was performed, and the compound was determined to have a molecular weight of 272.9733 with a likely structure of [$C_{11}H_7O_4Cl_2$-H]$^-$. Below, in Figure 11, is the proton NMR spectrum for the compound isolated from NASA 29. Figure 12 shown below is the carbon NMR from the same compound. Figure 13 below is a HSQC spectrum which is used to determine proton-carbon bond correlations. Finally, Figure 14 below is a HMBC spectrum which shows correlations between protons and carbons separated by two or three bonds. The final structure of this compound has not yet been solved but there is evidence that suggests that this compound will be new to the scientific community. It is unknown whether this compound is related to white-nose bat syndrome.

[8] Blehert, David S., et al. "Bat white-nose syndrome: an emerging fungal pathogen?." *Science* 323.5911 (2009): 227-227.

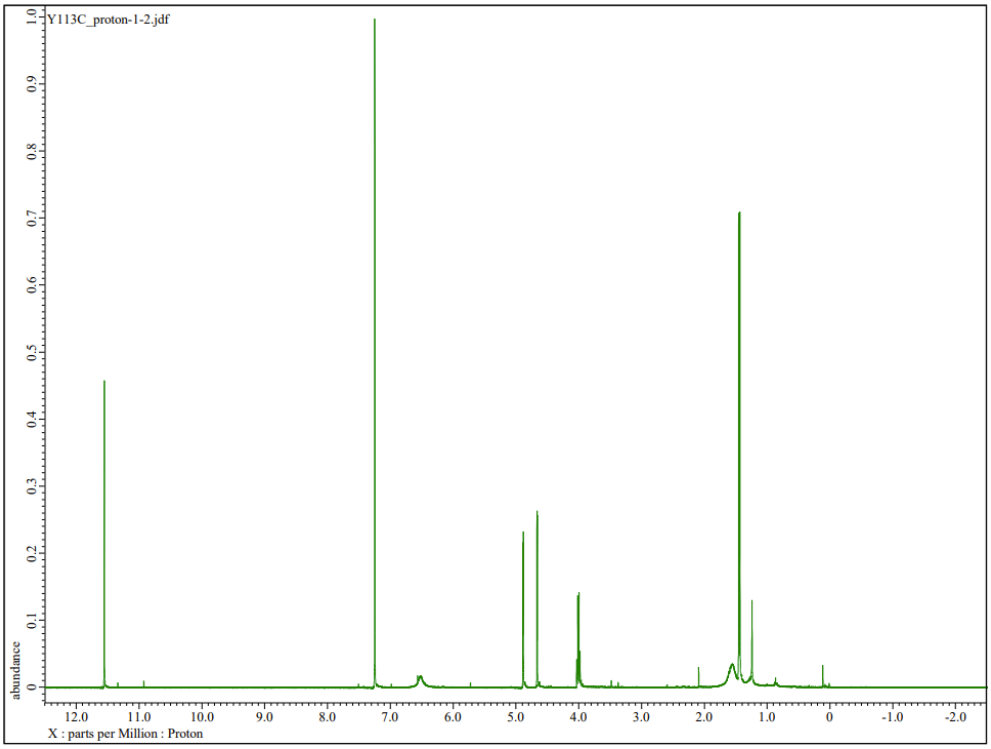

Figure 11. Proton NMR spectrum from NASA 29 compound

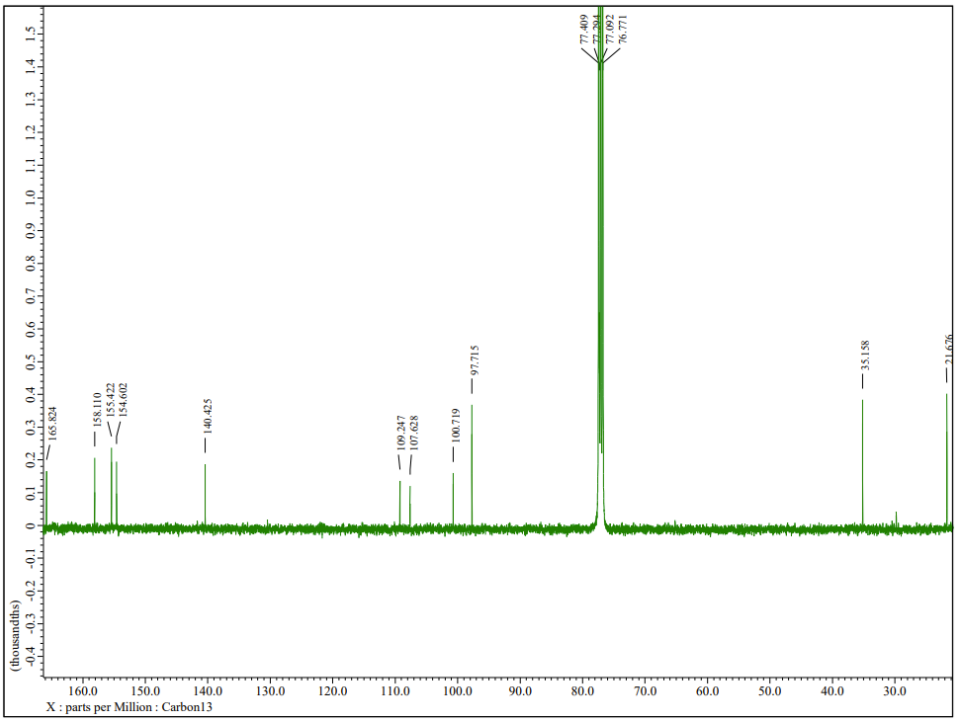

Figure 12. Carbon NMR spectrum from NASA 29 compound

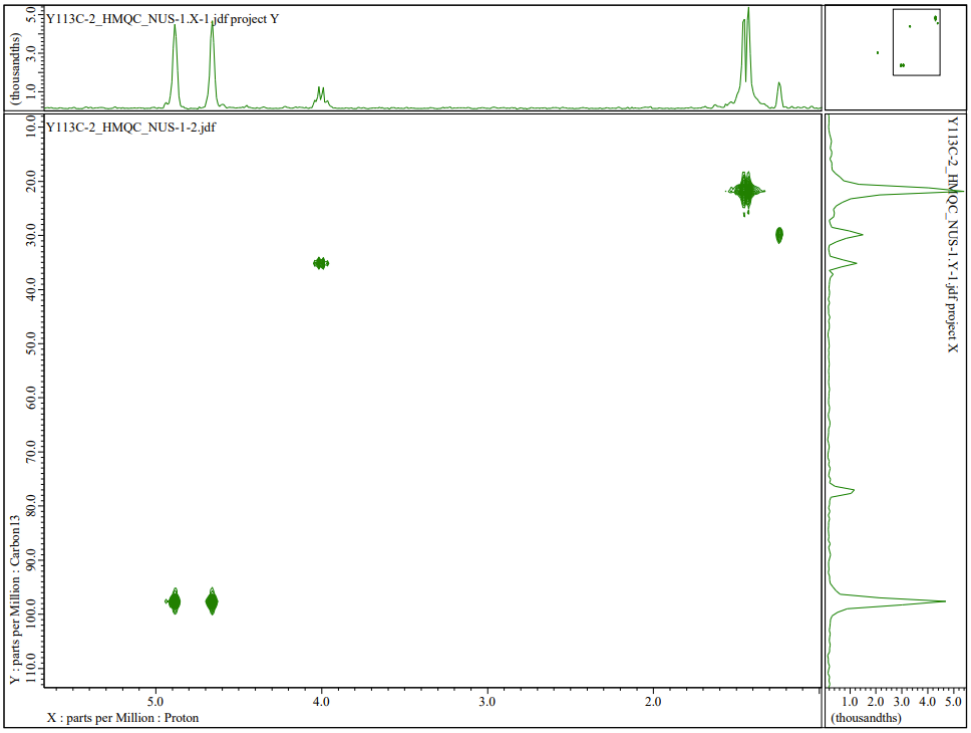

Figure 13. HMQC spectrum from NASA 29 compound

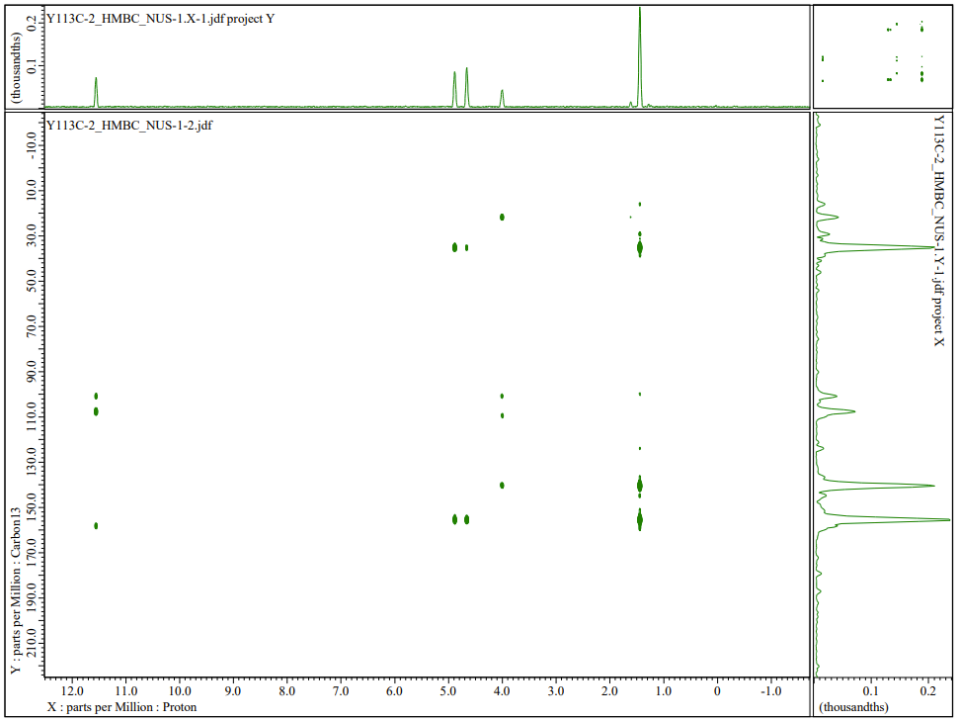

Figure 14. HMBC spectrum from NASA 29 compound

Conclusions

The purpose of this project was to identify fungal samples collected from Wind Cave, South Dakota. In addition, another goal of this project was to isolate and identify secondary compounds that showed antimicrobial growth inhibition so that they could be studied and potentially tested to be used as therapeutic drugs to combat bacteria. As a result of this project, eighteen unique fungal samples were isolated from water samples collected from Calcite Lake, a water source located within Wind Cave.

In respect to the DNA portion of the project, DNA from all eighteen of these samples was able to be successfully isolated and sequenced. The ITS region of the DNA did allow for successful identification of these samples to the genus level. One significant finding relating to the DNA isolation was the fact that two separate isolated fungal samples were found to belong to the *Pseudogymnoascus* genus. *Pseudogymnoascus destrucans* is a species from this genus that is known to cause a deadly disease in bats called white-nose syndrome. One potential shortcoming of exclusively using the ITS region of the fungal DNA to identify fungal samples is that it is difficult to confidently identify the species of samples. To confidently determine the species of the samples, more DNA will need to be sequenced from other 'barcode regions' of the fungal genome. This will allow for a better view of the genetic makeup of each sample and a higher likelihood of a more specific, and accurate, identification. The sample collected does seem to be a different species than *Pseudogymnoascus destrucans*, but it is unknown if it causes white-nose syndrome. It is important to know that fungi in the *Pseudogymnoascus* genus live in Wind Cave because of the potential harm they could cause to the wildlife that reside in the cave.

In respect to the secondary metabolite portion of the project, ten of the samples were successfully extracted and tested for antimicrobial growth, however enough sample was able to be isolated from the samples that exhibited growth inhibition to perform structural analysis for only one compound. This compound, called averantin, came from NASA 36, which is defined as a fungal sample belonging to the *Aspergillus* genus. The Aspergillus genus is a known producer of averantin. As of now there is no evidence that would support the development of a new pharmaceutical to combat antibiotic drug resistance amongst bacteria. Also of note is that fact that many of the isolated samples belonged to the *Penicillium* genus. There has been a great deal of exploration into the natural products of the *Penicillium* genus. This decreases the likelihood that new antimicrobial secondary metabolites will be identified in these samples. The other samples belonged to six different genera. The broad genus identification does not allow for thorough analysis of the literature to see if these samples, and the natural products they

produce, have been thoroughly studied. Thus, moving forward, work can be done to identify these samples further to see if research has been performed on each species and the secondary metabolites that they produce.

Moving forward, one important part of the research will be ensuring that results are reproducible in terms of what fungal samples were identified as living in Wind Cave. Though proper stereological measures were taken throughout the extraction and identification process, contamination of the fungal species could still have occurred during these steps or even during the initial collection in Wind Cave. Thus, to confidently determine that all the fungi, and the secondary metabolites, tested are from Wind Cave, more samples from the cave are required to confirm the results of this project as well as to collect more samples from the cave to better understand the fungal makeup. This project focused on only one area of water, Calcite Lake, in the vast cave. There are undoubtably more species that exist within this body of water as well as the many other areas in the cave.

This is an ongoing project and work will continue to be done to identify and extract secondary metabolites from fungal samples from Wind Cave. Collection of more samples allows for a greater view to the fungal makeup of the cave, as well as confirming the past collection experiments. This also allows for the potential of discovery of fungal species that are currently unknown by the scientific community. For the samples that were studied for this portion of the project, additional sample of the compounds that exhibited antimicrobial growth is required for confident structural analysis. Because the structure of the compounds that exhibited the antimicrobial properties remains a mystery and the one compound identified has already been discovered, there is currently no evidence that would warrant the development of a new pharmaceutical drug aimed to combat antibiotic resistance in bacteria.

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